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In re application of:

Donna G. Albertson; Daniel Pinkel; Antoine Snijders
Application No.: 09/574,386

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For: **Methods And Compositions For Preparation Of A Polynucleotide Array**

Examiner: Spiegler, A.

Art Unit: 1656

DECLARATION UNDER 37 C.F.R. 1.132

Commissioner for Patents
Alexandria, VA 22313

Dear Sir:

DECLARATION OF DR. DONNA G. ALBERTSON

I, Dr. Donna G. Albertson, am a Professor in Residence in the Cancer Research Institute and the Department of Laboratory Medicine at the University of California, San Francisco, and a co-inventor of the above-referenced patent application. I have read the above-referenced application and the currently pending claims. I have been asked to describe my expectations concerning the success of the method described and claimed in the patent application.

This method was developed in the course of my laboratory's efforts to prepare DNA microarrays from P1 or BAC DNA. We set out to prepare such microarrays by robotic spotting, using an array of pins or capillary dispensers that are dipped into wells, e.g., the 96 wells of a microtiter plate, for transferring an array of samples to a substrate. P1 or BAC clones are generally high molecular weight, with complexities up to about 200 kilobases, which makes P1 and BAC DNA solutions highly viscous and difficult to spot on microarray substrates.

Our initial effort to prepare P1 and BAC microarrays entailed using standard techniques to propagate and isolate P1 and BAC DNA to produce target solutions for spotting. We made no effort to reduce the molecular size of the P1 and BAC clones. It was possible to make and spot the DNA solutions by hand from small numbers of clones using these materials. However, the

procedures were too labor-intensive and resulted in too much variation in spotting for production of large numbers of arrays from thousands of clones.

We subsequently investigated other techniques aimed at reducing the viscosity of the P1 and BAC DNA target solutions and conveniently producing large amounts of DNA from the genomic clones. There are a wide variety of techniques that would reduce DNA molecular size, which would be expected to reduce target solution viscosity, facilitating spotting. However, to produce useful arrays, each target solution must be representative of the starting polynucleotide from which it was produced. More specifically, each target solution must, when spotted and hybridized with a labeled probe, produce a signal that is essentially the same as the signal obtained from the starting polynucleotide.

We decided to test the following three techniques for producing P1 or BAC DNA target solutions: (1) fragmenting the P1 or BAC DNA using sonication or chemical treatments of the DNA; (2) ligation-mediated polymerase chain reaction (PCR); and (3) "shotgun cloning" the P1 or BAC inserts into a DNA sequencing vector, followed by PCR amplification of the ligation mixture. In advance of these studies, we could not predict whether any of these techniques would yield sufficiently representative target solutions. In particular, we could not be sure that the amplification-based techniques would satisfy this requirement, as essentially all of the starting P1 BAC DNA sequences would have to be amplified to essentially the same extent to produce an amplification product (and, ultimately, target solution) in which the P1 or BAC DNA sequences were present in approximately the same proportions as in the starting P1 or BAC DNA.

Prior to carrying out these studies, my expectation was that the third approach, shotgun cloning, followed by PCR, was the most likely to give satisfactory results. In fact, as detailed below, this approach failed absolutely. However, the second approach, based on ligation-mediated PCR, worked unexpectedly well. Each of these approaches and the results obtained are described below.

Fragmenting of P1 or BAC DNA

In order to facilitate spotting the viscous solutions of high molecular weight BAC DNA, we reduced the size of the DNA by shearing. The DNA from each BAC (10 μg), in a total volume of 200 μl, was sonicated 3 times for 1 sec. at a sonication output of 65 using a Kontes Micro Ultrasonic Cell Disrupter. BAC DNA was ethanol precipitated using 2 volumes of 100% ethanol and 1/10 volume

of sodium acetate (3 M). The precipitate was collected by centrifugation at 14,000 rpm at 4°C. The BAC DNA pellet was dissolved in 5 µl of a 20% dimethylsufoxide (DMSO) solution.

The resulting solutions were then hand-spotted on a silylated glass slide, using a small capillary. The slide was dried overnight at room temperature, and target DNA was linked to the slide using a UV Stratalinker 2400™ (2 treatments each at 65 mJoules).

A square of rubber cement was applied around each spot. 40 µl FITC-labeled BT474 (human tumor) DNA (approx. 500 ng), 40 µl TR-labeled normal human DNA (approx. 500 ng), and 100 µl Cot-1 DNA (approx. 50 µg) was precipitated by adding 2 volumes of ice-cold ethanol and 0.1 volume of NaOAc (3 M, pH 7) and centrifuging for 30 min. at 14,000 rpm. After removing the supernatant and air-drying the pellet, the pellet was resuspended in 7 µl master mix (1 g dextran sulfate, 5 ml formamide, 1 ml 20xSSC, water to bring total volume to 7 ml), 2 µl 20% SDS, and 1 µl tRNA (100 µg/µl, Boehringer Mannheim). The probe mixture was denatured at 70°C for 10 min. and then pre-incubated at 37°C for 1 hr. After the pre-incubation, the probe mixture was applied on the spot inside each rubber cement square.

Hybridization was carried out for 2 nights at 37°C on a rocking table in a humid chamber containing 50% formamide, 2xSSC in water.

After hybridization, the slide was washed for 15 min. at 45°C in a mixture containing 50% formamide 2xSSC in water. This washing procedure was repeated in fresh wash solution. The slide was then washed for 20 min. in phosphate-buffered saline (PBS) containing 0.05% Tween after which the rubber cement was removed from slide. The slide was washed for 5 min. in fresh PBS containing 0.05% Tween. Finally after air drying the slide, it was sealed in 25 µl DAPI/Antifade solution. The result was evaluated using a Zeiss fluorescence microscope, equipped with three separate band pass filters (DAPI, FITC and TR).

The sonicated BAC DNA target spots were of good quality and the solutions were easier to spot than prior to sonication due to the reduced viscosity.

Ligation-Mediated PCR of P1 or BAC DNA

For ligation-mediated PCR of P1/BAC DNA, we digested the DNA with a *Mse I* restriction enzyme, which leaves a TA overhang for attachment of adapter sequences to both ends of each fragment. PCR was then carried out using primers that anneal to the adapters.

In particular, BAC DNA was digested with *Mse I* in a 5 µl reaction volume containing: 2 .2x One-Phor-All Buffer Plus™, 2 U *Mse I* restriction enzyme and 20-500 ng BAC DNA. The digestion was set up as follows:

1. Dilute the 10x One-Phor-All Buffer Plus™ to a final concentration of 0.8x in a volume of 750 µl using sterile H_2O.

2. Dispense 2.5 µl of the 0.8x buffer solution into each tube of an 8-tube strip and seal the tubes to prevent evaporation and possible contamination.

3. Add 1.5 µl BAC DNA to each tube using a single-channel pipettor. The BAC DNA concentration usually ranges from 20-400 ng/µl.

4. Dilute the *Mse I* restriction enzyme to a final concentration of 2 U/µl in a volume of 60 µl using 10x One-Phor-All Buffer Plus™. Keep the enzyme and the dilution on ice during this process.

5. Dispense 1 µl (2 U) of the *Mse I* enzyme dilution into each tube individually; the enzyme should stay on ice during this process. Cap the 8-tube strip using an 8-cap strip after adding the enzyme, and place on ice.

The reaction was placed in a PCR machine for an overnight incubation at 37°C (12-16 hours). After digestion, 1.75 µl of the digests were run on a conventional 1% agarose gel containing 0.5 µg/ml ethidium bromide along with a φ X174 RF DNA/Hae III Marker to check the distribution of fragment lengths. 1 µl of each of the restriction digests from the preceding step was diluted to 1 ng/µl using sterile H_2O.

Primers were ligated to digested BAC DNA in a 10 µl reaction volume containing: 5 µM Primer 1, 5 µM Primer 2, 0.5x One-Phor-All Buffer Plus™, 1 mM ATP, 5 U T4 DNA ligase and 1 ng digested BAC DNA.

The ligation was set up as follows:

Primer Solution:

Primer 1 (100 µM) (OPERON)	28 µl
Primer 2 (100 µM) (OPERON)	28 µl
One-Phor-All Buffer Plus™	28 µl
H_2O	308 µl

Total	392 µl

1. Mix the above-prepared primer solution by pipetting, and dispense 7 µl of primer solution onto the bottom of new 8-tube strips.

2. Add 1 µl of the 1 ng/µl BAC DNA digest prepared above to the 7 µl primer solution in each corresponding tube of the 8-tube strip.

3. Put the 8-tube strips into a PCR machine. The annealing reaction is carried out at 65°C for 1 min., then the temperature is shifted down to 15°C, with a ramp of ~1.3°C/min. (in a Perkin-Elmer 9700 PCR machine this is a ramp rate of 5%).

4. As soon as the PCR machine reaches 15°C, promptly take out the tubes from the PCR machine and carefully open all 8-tube strips . Using a single-channel pipettor, dispense 1 µl of 10mM ATP solution on the inside wall of each of the tubes containing DNA. Gently tap the PCR rack so that the ATP slides into the DNA/primer solution.

5. Dispense 1 µl (5 U) of the T4 DNA ligase enzyme into each of the tubes containing DNA. Cap the 8-tube strip using an 8-cap strip after adding the enzyme and place on ice.

The ligation reaction was placed in a PCR machine for an overnight incubation at 15°C (12-16 hours).

After ligation of primers to the BAC DNA, ligation-mediated PCR was carried out in a reaction volume of 50 µl containing: 0.6x 10xPCR buffer #1, 0.4 mM dNTP mixture, 3.5 U DNA polymerase mixture, and 10 µl ligation mixture .

The PCR was set up as follows:

Diluted dNTP Mixture:

10xPCR buffer #1	168 µl
dNTP mixture (10 mM)	112 µl
H_2O	1960 µl
Total	2240 µl

1. Mix the diluted dNTP mixture briefly by vortexing and place on ice.

2. Remove the ligations prepared above from the PCR machine. Open the 8-tube strips carefully. Using a single-channel pipettor dispense 40 µl of the above-prepared dNTP mixture into each tube of the 8-tube strips. Gently tap the PCR rack so that the diluted dNTP mixture slides into the DNA/primer solution. Cap with 8-cap strips.

3. To melt off Primer 2, place PCR tubes in the PCR machine at 68°C for 4 min. After 4 min., take out the tubes and add 1 µl of DNA polymerase mixture (3.5 U/µl) to each tube individually. Cap immediately after adding the enzyme mix, and place on ice.

4. Place the 8-tube strips in the PCR machine: 68°C for 3 min.; 94°C for 40 sec., 57°C for 30 sec., 68°C for 1 minute 15 sec. for 14 cycles, 94°C for 40 sec., 57°C for 30 sec., 68°C for 1 minute 45 sec. for 34 cycles and 94 °C for 40 sec., 57°C for 30 sec. and 68°C for 5 min. for the last cycle, followed by incubation at 4°C.

After PCR, 3.5 µl of each reaction was run on a conventional 1% agarose gel containing 0.5 µg/ml ethidium bromide along with a φX174 RF DNA/Hae III Marker to determine the distribution of fragment lengths.Each PCR product can then be reamplified to increase the amount of P1/BAC DNA for a target solution. Reamplification was carried out as follows.

The primary PCR products have a MseLig 21 sequence on the 5' side. For re-amplification, the MseLig21 primer was used (OPERON) (5'-AGT GGG ATT CCG CAT GCT AGT-3'; containing a 5' aminolinker).

PCR set-up:

MseLig 21 (100 µM) (OPERON)	4 µl
10x TAQ-buffer II (contains no $MgCl_2$) (Perkin Elmer)	10 µl
Primary PCR DNA (approximately 100 ng/µl)	0.5 µl
dNTP mix (25 mM) (Boehringer Mannheim)	0.8 µl
Amplitaq Gold™ (5 units/µl) (Perkin Elmer)	0.5 µl

$MgCl_2$ (25 mM) (Perkin Elmer)	22 µl
H_2O	62.2 µl
Total for one PCR	100 µl

Thermal cycling was carried out in a Perkin-Elmer Gene Amp™ PCR system 9700 block as follows: activation of Amplitaq Gold™ Polymerase at 95°C for 10 min., denaturation at 95°C for 30 sec., annealing at 50°C for 30 sec., polymerization at 72°C for 1 min. 15 sec., for 45 cycles, followed by 7 min. at 72°C.

After PCR, the DNA concentrations were measured using a fluorimeter after which the PCR products were separated on a 1% Seakem LE (FMC Bioproducts) agarose gel containing 0.5 µg/ml ethidium bromide in TBE together with a *φX174 RF DNA/Hae* III Marker (Gibco BRL) to check the distribution of fragment lengths.

Between 5 and 10 µg of DNA was precipitated by adding 1/10 volume of 3 M NaOAc and 2 volumes of cold 100% Ethanol. After mixing well, DNA was precipitated by centrifuging at 14,000 rpm at 4°C for 30 min. After removing the supernatant, the pellets were resuspended in 500 µl 70% ethanol and sedimented again at 14,000 rpm at 4°C for 30 min., after which the supernatant was removed carefully. After air-drying, the pellets were dissolved in 1 µl H_2O and 4 µl 1% nitrocellulose in DMSO.

Each P1/BAC DNA target solution was then hand-spotted onto a slide, side by side with the starting P1/BAC DNA for each target solution (i.e., the corresponding P1/BAC clone DNA prepared by growing the clone, isolating and then sonicating the DNA), as described above. The slide was hybridized with FITC-labeled BT474 (human tumor) DNA, TR-labeled normal human DNA, and Cot-1 DNA, as described above.

After hybridization and two-color fluorescent detection, the signal intensity ratios (i.e., tumor DNA signal intensity divided by normal DNA signal intensity) were essentially the same (within a few percent) for targets produced by ligation-mediated PCR as for the targets containing the starting P1/BAC DNA clones. These data indicated that the target solutions produced by ligation-mediated PCR were highly representative of the starting P1/BAC clones from which the target solutions were prepared. These results demonstrated that the spots made with the ligation-

mediated PCR procedure performed equivalently to spots made directly from BAC DNA, so that the ligation-mediated PCR adequately represented the sequences in the BAC for array CGH. The target solutions were superior to solutions of starting P1/BAC DNA in two respects. Very small volumes of target solutions prepared by ligation-mediated PCR could be spotted robotically, and amplification produced large amounts of target DNA more readily than propagation of P1/BAC clones, followed by isolation of P1/BAC DNA from host cells.

Shotgun Cloning and PCR of P1 or BAC DNA

Shotgun cloning of P1/BAC DNA was carried out by first sonicating the P1/BAC DNA to randomly shear the DNA to an approximate size of 1 kb (see above). The resulting fragments have single-stranded overhangs that are made blunt-ended by treatment with either mung bean nuclease, an exonuclease breaking single-stranded DNA in either direction (5' to 3' and 3' to 5'), or T4 DNA polymerase, a DNA polymerase that catalyzes the polymerization of deoxynucleoside-5'-triphosphates to the hydroxyl termini of recessive ends and carries an extremely active 3' to 5' exonuclease activity. We ligated the blunt fragments into a Plasmid Bluescript-SK™ (PB-SK) using T4 DNA ligase, after cutting the plasmid with a *Sma-1* restriction enzyme, leaving blunt ends, and phosphatase-treating these blunt ends using calf intestinal alkaline phosphatase (CIAP). After ligation, the PB-SK was transfected into *E. coli* bacteria strain TOP 10 F'. Thus, for every different P1/BAC clone, a bacterial mixture was generated that contained plasmids having random P1/BAC insert fragments. To make spotting solutions, PCR was carried out directly on the bacteria, since the denaturing step in the PCR protocol lyses the bacteria, freeing the plasmid for subsequent primer annealing and elongation. In addition, to check for successful ligation, an aliquot of the mixture was plated on a LB plate containing ampicillin (for selection of transfected bacteria) and IPTG and X-GAL (for selection based on presence of an insert in the plasmid), after which positive (white) colonies were picked and subjected to PCR. The experimental details of this procedure are as follows.

To prepare P1/BAC inserts for cloning into PB-SK, 10 µl of P1/BAC DNA, sonicated as described above, was run on a 1% Seakem LE (FMC Bioproducts) agarose gel containing 0.5 µg/ml ethidium bromide in TBE together with a *φX174 RF DNA/Hae* III Marker (Gibco BRL) to confirm a fragment length distribution around 1 kb.

To make the fragment ends blunt, we treated the fragments with either mung bean nuclease or T4 DNA polymerase. For the mung bean nuclease treatment, 90 µl of the sonicated DNA was mixed with 10 µl mung bean nuclease buffer (10x; Promega) and 0.5 µl mung bean nuclease (60 units/µl; Promega) and incubated at 37°C for 10 min. For the T4 DNA polymerase treatment, 76 µl of the sonicated DNA was mixed with 20 µl T4 DNA polymerase buffer (5x; Boehringer Mannheim), 2 µl T4 DNA polymerase (1U/µl; Boehringer Mannheim), and 4 µl dNTP mix (10mM; Gibco BRL) and incubated at 37°C for 30 min.

Both mixtures were run over a microcon YM50 (Millipore) to desalt the DNA and separate it from mung bean nuclease (if present) and DNA fragments shorter than 100 bp, according to the manufacturer's instructions. 100 µl water was then run over the microcon the same way as the DNA mixture, to wash the filter for any residual salt or enzyme. The DNA was eluted two times from the microcon filter using 5 µl water each time, following the manufacturer's instructions.

The PB-SK vector was prepared by digesting with *Sma-1*, followed by phosphatase treatment. Specifically, 2 µl PB-SK (2 µg/µl) was mixed with 6 µl H_2O, 1 µl *Sma-1* (10 U/µl), and 1 µl reaction buffer 4 (10x), and incubated at 37 °C for 60 min., leaving blunt ends. The digest product was run on a 1% Seakem LE (FMC Bioproducts) agarose gel containing 0.5 µg/ml ethidium bromide in TBE. The band was cut out on a UV-lightbox. DNA was isolated from the agarose using a Qiaex II Agarose Extraction Kit (Qiagen) according to the manufacturer's instructions, using 20 µl H_2O to elute the DNA. For phosphatase treatment, 2 µl Calf Intestine Alkaline Phosphatase (CIAP) (1 unit/µl; Gibco BRL) and 2 µl CIAP buffer (10x; Gibco BRL) were added to the PB-SK DNA solution and incubated at 50°C for 60 min. The mixture was then incubated at 70°C for 10 min. to inactivate the CIAP. The P1/BAC DNA fragment mixture for each clone was then added to a ligation mixture containing the PB-SK vector.

Ligation Set-up:

T4 ligase (5 units/µl) (Gibco BRL)	0.5 µl
10x T4 ligase buffer (Gibco BRL)	4 µl

PB-SK (vector)	60 fmol
P1/BAC DNA fragments (insert)	180 fmol
H_2O	to 20 µl

The reaction was incubated overnight at 14°C.

Each ligation mixture was transfected into the *E. coli* bacteria strain TOP 10 F' (purchased from Invitrogen in a One Shot™ format (50µl)). 5 µl ligation mixture and 1.6 µl β-mercapto-ethanol were mixed with one shot of TOP 10 F' *E. coli* bacteria and incubated at 4 °C for 30 min. Subsequently, the *E. coli* bacteria were heat-shocked at 42°C for 30 sec., after which the sample was incubated at 4°C for 2 min. 250 µl S.O.C. medium was added to the mixture (10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose; Gibco BRL) after which the mixture was incubated in a shaker at 37°C for 60 min. at 225 rpm.

To check for successful ligation and transformation, 20 µl bacteria mixture was plated on a LB plate (5g tryptone (Difco), 2.5 g yeast extract (Difco), 2.5 g NaCl, 0.5 ml 1M NaOH and 7.5 g bacto agar (Difco) in 0.5 ml of water) containing ampicillin, IPTG and X-GAL at a concentration of 25 mg/ml, 0.1 mM and 20 µg/ml, respectively. The plates were incubated overnight at 37°C.

PCR was carried out on white colonies, picked from the LB plates with a toothpick, by rubbing the toothpick against the wall of the PCR tube containing the PCR mixture. For PCR, T3-and T7-specific primers were used, which were aminolinked at the 5' end (OPERON) (T3; 5'-AAT TAA CCC TCA CTA AAG GG-3' and T7; 5'-TAA TAC GAC TCA CTA TAG G-3').

PCR Set-up:

Primer T3-5'aminolinked (20 µM) (OPERON)	1 µl
Primer T7-5'aminolinked (20 µM) (OPERON)	1 µl
10x TAQ-buffer II (contains no $MgCl_2$) (Perkin Elmer)	5 µl
Tooth picked white colonies	-

dNTP mix (25 mM) (Boehringer Mannheim)	0.4 µl
Amplitaq Gold (5 units/µl) (Perkin Elmer)	0.25 µl
$MgCl_2$ (25 mM) (Perkin Elmer)	11 µl
H_2O	31.35 µl
Total for one colony	50 µl

Thermal cycling was carried out in a Perkin-Elmer Gene Amp. PCR system 9700 block as follows: activation of Amplitaq Gold Polymerase at 95°C for 10 min., denaturation at 95°C for 30 sec., annealing at 50°C for 30 sec., polymerization at 72°C for 1min. 15 sec., for 45 cycles, and finally 7 min. at 72°C. After PCR, the products were separated on a 1% Seakem LE (FMC Bioproducts) agarose gel containing 0.5 µg/ml ethidium bromide in TBE together with a *φX174 RF DNA/Hae* III Marker (Gibco BRL) to check presence and size of the insert.

To make spotting solutions from the *E. coli* bacteria mixtures, T3- and T7-specific primers were used, aminolinked at the 5' end (OPERON) (T3; 5'-AAT TAA CCC TCA CTA AAG GG-3' and T7; 5'-TAA TAC GAC TCA CTA TAG G-3').As template, we used the generated *E. coli* bacteria mixture containing plasmids with inserts, which are made blunt ended by either mung bean nuclease or T4 DNA polymerase.

Primer T3-5'aminolinked (20 µM) (OPERON)	1 µl
Primer T7-5'aminolinked (20 µM) (OPERON)	1 µl
10x TAQ-buffer II (contains no $MgCl_2$) (Perkin Elmer)	5 µl

Bacteria mixture containing plasmids with mung bean nuclease/T4 DNA polymerase treated inserts	0.5 µl
dNTP mix (25 mM) (Boehringer Mannheim)	0.4 µl
Amplitaq Gold (5 units/µl) (Perkin Elmer)	0.25 µl
$MgCl_2$ (25 mM) (Perkin Elmer)	11 µl
H_2O	30.85 µl
Total for one mixture	50 µl

Thermal cycling was carried out in a Perkin-Elmer Gene Amp. PCR system 9700 block as follows: activating Amplitaq Gold Polymerase at 95°C for 10 min., denaturing 95°C for 30 sec., annealing 50°C for 30 sec., polymerization 72°C for 1 min. 15 sec., for 45 cycles and finally 7 min. at 72°C. After PCR, the products were separated on a 1% Seakem LE (FMC Bioproducts) agarose gel containing 0.5 µg/ml ethidium bromide in TBE together with a *φ X174 RF DNA/Hae* III Marker (Gibco BRL) to check fragment length.

DNA was precipitated by adding 1/10 volume of 3 M NaOAc and 2 volumes of cold 100% ethanol, after mixing well, DNA was precipitated at 14,000 rpm at 4°C for 30 min. After removing the supernatant, the pellets were resuspended in 500 µl 70% ethanol and sedimented again at 14,000 rpm at 4°C for 30 min., after which the supernatant was removed carefully. After all pellets were air-dried they were dissolved in 1 µl H_2O and 4 µl 1% nitrocellulose in DMSO.

Each P1/BAC DNA target solution was then hand spotted onto a slide, side by side with the starting P1/BAC DNA for each target solution (i.e., the corresponding P1/BAC clone DNA prepared by growing the clone and isolating the DNA), as described above. The slide was hybridized with FITC-labeled BT474 (human tumor) DNA, TR-labeled normal human DNA, and Cot-1 DNA, as described above.

After hybridization and two-color fluorescent detection, ratios obtained on BAC spots prepared from BAC DNA and from the PCR-amplified DNA from the bacterial ligation mixtures

were compared. Hybridizations comparing BT474 breast cancer cell line DNA to normal reference DNA were carried out on two separate days and, in both cases, the hybridization intensities were very low on the spots made from the DNA amplified from the ligation mixtures, and the calculated fluorescence ratios were very different from the ratio obtained on the whole BAC DNA as shown in the Table below.

Hybridization Date	11/18/99	12/08/99
Mean Background-Corrected Fluorescence Intensity (Green) B4130 Whole BAC DNA	2786.8	631.1
Mean Background-Corrected Fluorescence Intensity (Red) B4130 Whole BAC DNA	1688	701.8
Raw Ratio B4130 Whole BAC DNA	1.65	0.90
Mean Bkgd-Corr. Fluor. Intensity (Green) B4130 Ligation Mix Prepared with T4 Ligase	95.2	24.5
Mean Bkgd-Corr. Fluor. Intensity (Red) B4130 Ligation Mix Prepared with T4 Ligase	266.2	102
Raw Ratio B4130 Ligation Mix Prepared with T4 Ligase	0.36	0.24
Mean Bkgd-Corr. Fluor. Intensity (Green) B4130 Ligation Mix Prepared with Mung Bean Nuclease	15.3	6.8
Mean Bkgd-Corr. Fluor. Intensity (Red) B4130 Ligation Mix Prepared with Mung Bean Nuclease	53.6	39.4
Raw Ratio B4130 Ligation Mix Prepared with Mung Bean Nuclease	0.30	0.18

Conclusion

In summary, when faced with the problem of how to prepare target solutions from high molecular weight DNA clones that could be robotically spotted on a substrate to form a DNA microarray, we realized that we could reduce target solution viscosity, facilitating robotic spotting,

by reducing the molecular size of the DNA in the target solutions. This goal could be achieved in a wide variety of ways, but to produce useful arrays, the technique chosen had to produce target solutions that were representative of the starting polynucleotide from which each target solution was produced. We decided to test three techniques for producing BAC DNA target solutions, namely: (1) fragmenting the P1 or BAC DNA; (2) ligation-mediated PCR; and (3) "shotgun cloning" the P1 or BAC inserts into a DNA sequencing vector, followed by PCR. In advance of these studies, we could not predict whether any of these techniques would yield sufficiently representative target solutions. In particular, we could not be sure that the amplification-based techniques would satisfy this requirement. Prior to carrying out these studies, I believed that the third approach, shotgun cloning followed by PCR, was the most likely to give satisfactory results. In fact, this approach failed, yielding targets prepared from human DNA that appeared incapable of hybridizing to human DNA as indicated by the low fluorescence intensity of the hybridization to these spots. This result demonstrates the difficulty in this field of predicting what technique will work for a particular application, based only on a theoretical understanding of the technique and/or information about its suitability for different applications.

Contrary to results obtained with the shotgun cloning approach, the ligation-mediated PCR approach worked unexpectedly well, producing target solutions that, when spotted and hybridized with a labeled probe, produced a signal that is essentially the same as the signal obtained from the starting polynucleotide. As the failure of the shotgun cloning approach demonstrates, this result could not have been predicted based on the available information concerning ligation-mediated PCR.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declarant's signature:

Donna G. Albertson

Donna G. Albertson, Ph.D.

Oct. 14, 2003

Date